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One of the fundamental aspects of malignant cells is their ability to invade the extracellular matrix. This capability is dependent on the elaboration of secreted and membrane bound proteases at the invasion front (invadopodia). Seprin is a 170 kDa integral membrane gelatinase that has been reported to be localized on invadopodia of metastatic LOX melanoma cells. We have screened a LOX cDNA library and isolated 10 seprin cDNA clones which range in size from 0.5 to 1.8 kb. Restriction endonuclease digestions of these clones indicate that 2 overlapping clones, lambda 50A2 and lambda 30B1 should encode the entire open reading frame for the 97 kDa monomeric subunit of seprin. Preliminary DNA sequence analysis indicates that seprin is homologous to the Fibroblast Activation Protein (FAP) which is reported to be expressed on active fibroblasts in the stroma of breast carcinomas and other carcinomas. However, we detect seprin/ FAP mRNA in the breast carcinoma cell line MDA - 436.

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Introduction

Background

Degradation of the extracellular matrix (ECM) is a normal component of tissue homeostasis. Indeed, normal physiological processes such as angiogenesis, wound healing, cartilage and bone repair, morphogenesis and macrophage migration involve degradation of proteins, proteoglycans and glycosaminoglycans present in the basal laminae or ECM. However, the ability to degrade the ECM is also a key requirement for the dissemination of malignant cells from a primary tumor to secondary sites in the body. Specifically, the production of various secreted and membrane associated proteases are required to degrade adhesive (e.g. fibronectin, laminin) and structural (e.g. collagen, proteoglycans) proteins. This allows neoplastic cells to invade or migrate through basement membranes and interstitial stroma and thus gain access to and allow extravasation from the bloodstream and/or the lymphatic system for dissemination to new host organs (Liotta et al., 1991; Monsky and Chen, 1993). For example, in advanced breast cancer metastasis to lung, liver, lymphatic organs and bone etc are common. And several proteases including type IV collagenase, stromelysins, cathepsin D and urokinase plasminogen activator are thought to have roles in this process (Chen et al., 1994).

Our laboratory (Chen, 1984) and others (David-Pfeuty and Singer, 1980; Tarone et al., 1985) discovered that transformed cells have extracellular, ventral plasma membrane protrusions, invadopodia, that were initially termed " rosettes " or " podosomes " respectively. They were thought to represent cell - matrix adhesion sites, however, it is now known that invadopodia are associated with cell surface protease activity (s) that degrades the ECM (Kelly et al., 1994; Monsky et al., 1993). Although invadopodia are formed on some normal cells including macrophages and osteoclasts (Marchisio et al., 1987) in general there appears to be a direct correlation between invasiveness or metastatic capability and the elaboration of these specialized structures at the invading front --- sites of cell invasion into the ECM (Kelly et al., 1994).

Using an *in vitro* assay - that consists of flourescently labelled ECM components that are covalently crosslinked to gelatin films (Chen et al., 1984) our laboratory identified a highly invasive melanoma cell line, LOX, from over 32 human cell lines tested (Aoyama and Chen, 1990). Recently, using this assay system two hormone independent breast carcinoma cell lines MDA - MB - 231 and MDA - 436 were also identified as having invasive or metastatic potential (Chen et al., 1994 ; see Body of Report).

LOX melanoma cells express a neutral 170 kDa membrane gelatinase (Seprin) that has been reported to be localized on invadopodia (Monsky et al., 1994). This 170 kDa gelatinase is a homodimer consisting of two noncovalently linked 97 kDa subunits which are inactive as monomers. It was subsequently discovered that seprin is not unique to LOX cells but is also present in human term placenta (Lin, 1994). Two

forms of seprin have been shown to exist in placental tissues: one form appears to be very similar in size and subunit composition to the 170 kDa gelatinase from LOX while the second form is a heteromeric protein containing a 95 kDa seprin subunit that is noncovalently linked to a 105 kDa subunit that has been identified as dipeptidyl peptidase IV (DPP IV).

Preliminary protein sequence data obtained from 3 Lys - C peptide fragments from LOX(97 kDa) and placenta (95 kDa) monomeric subunits indicated sequence homology to the fibroblast activation protein (FAP ; Lin, 1994; Rettig et al., 1994; Scanlan et al., 1994). This protein (95 kDa) shows a restricted pattern of expression in adult humans being limited to activated fibroblasts in wound healing and pancreactic islet A cells (Rettig et al., 1988). It has been detected in established tumor cell lines of various sarcomas and some melanomas. However, the most striking pattern of expression is in carcinomas such as breast cancer etc where expression is restricted to activated stromal fibroblasts but not the malignantly transformed epithelial cells (Garin - Chesa et al., 1990). The full length cDNA sequence of FAP has been obtained. It shows significant sequence homology to two closely related genes: DPP IV with a 61% nucleotide sequence identity and a 48% amino acid sequence identity and dipeptidyl peptidase related protein (DPP X) with a 30% amino acid sequence identity (Wada et al., 1992). Like DPP IV it is a type II integral membrane protein and a nonclassical serine protease. In addition , like seprin it forms a heteromeric complex with DPP IV.

Purpose of present work

In the initial grant proposal our goal was to determine the role of the 170 kDa gelatinase (seprin) in the metastasis of breast cancer by the induction or restriction of seprin cDNA sense and antisense constructs in breast cancer cell lines at various stages of neoplastic development. The results presented above suggest that FAP and seprin are homologous closely related proteins. The fact that homomeric seprin contains a 97 kDa subunit as opposed to 95 kDa for FAP suggests that their may be a difference in their primary structures. Therefore, our first priority is to isolate a full length seprin cDNA from LOX cells. Because of the apparent close identity between the two proteases we will use oligonucleotides based on the FAP cDNA sequence to generate probes from RNA polymerase chain reaction (PCR) to screen a LOX cDNA library.

Body of Report

Six oligonucleotides (3 sense primers and 3 nonsense primers) were synthesized based on the FAP cDNA sequence (Scanlan et al., 1994). This would allow using RNA PCR the production of amplicons that included the entire open reading frame (ORF) of the FAP cDNA corresponding to nucleotides # 209 through #2488. The sequence (5' to 3') and the corresponding nucleotide position of the six oligonucleotides were: 1) FAP - 1 (sense) CCACGCTCTGAAGACAGAATT # 161 - 181; 2) FAP - 2 (nonsense) GATTCTTAGCTCCAGCCT # 986 - 969; 3) FAP - 3 (sense) CCAGCAATGATAGCCTCAA # 1055 - 1073; 4) FAP - 4 (nonsense) ACAGACCTTACACTCTGAC # 1863 - 1845; 5) FAP - 5 (sense) TGACAAACTCCTCTATGCAG # 1951 - 1970; and 6) FAP - 6 (nonsense) TCAGATTCTGATACAGGCT # 2523 - 2505. Thus the oligonucleotides FAP - 1 and FAP - 6 correspond to sequences within the 5' and 3' untranslated regions (UTR) and FAP - 2,3,4 and 5 correspond to sequences within the ORF.

The amplicon generated with primers FAP - 3 and 4 (0.8 kb fragment corresponding to nucleotides # 1055 - 1863) was used as a probe to screen a lambda gt11 cDNA library made from LOX mRNA (Sambrook et al., 1989). It should be noted that we chose to screen a cDNA library instead of sequencing directly the RNA PCR products generated with FAP primers since Taq polymerase lacks 3' proof reading capability and therefore might produce artifactual sequences. Approximately 150,000 plaques were screened and 13 recombinant phages were isolated that gave a reproducible positive signal. Insert sizes of 12 of the 13 phages were estimated by producing PCR amplicons with lambda gt11 forward and reverse primers that border the unique EcoR I insertion site. Inserts sizes ranged from 0.5 to 1.8 kb. Thus none of the clones represents a full length cDNA nor does any one clone encode the entire ORF. A restriction map was generated based on the FAP cDNA sequence. Restriction endonucleases Dra I (351 - nucleotide cut site), Pst I (424, 1844), Sca I (438, 1581), Xba I (547), Hind III (566), Cla I (1008), EcoR V (1881), EcoR I (2239) and Ase I (2600) were utilized for restriction digests of the phage amplicons (except for Ase I all restriction sites are within the sequence encoding the ORF). Restriction mapping indicated that 10 clones were homologous to FAP cDNA. Two clones lambda 50A2 (1.8 kb) and lambda 30B1 (1.4 kb) overlapped each other within the FAP sequence # ~1300 - 1850. Based on their insert sizes and restriction maps we can predict that they should encode the entire ORF and therefore were choosen for DNA sequence analysis.

The cDNA inserts of lambda 50A2 and 30B1 were subcloned into the pUC19 and pLitmus 28 plasmid vectors. Using M13 forward and reverse primers and the FAP primers we have recently begun to sequence these clones. The initial sequence data confirms the restriction maps; seprin and FAP are homologous (nucleotides # 155 - 299 show a 100% identity and encode FAP amino acid residues # 1 - 30) and the exact degree of homology will be determined in the very near future.

Since one of our goals is to carry out transfections of normal and neoplastic breast cancer cells with sense and antisense seprin cDNA constructs a full length cDNA is required. Restriction digests and DNA sequence analysis indicate a unique BspH I site at nucleotide position #1635 exists in the overlap region of the 2 clones. Thus it should be possible to ligate the clones and generate a full length cDNA. Alternatively, RNA PCR using LOX RNA and LOX/FAP primers should also produce a full length cDNA.

Two metastatic breast cancer cell lines , MDA - MB 231 and MDA - 436 (see Introduction) were assayed for the presence of FAP/seprin mRNA using FAP primers (see above) by RNA PCR. Results of preliminary qualitative experiments suggests that MDA - 436 has a level of FAP/seprin mRNA equivalent to LOX melanoma cells. However, MDA - MB - 231 appears to have little or none. Therefore, we plan to use antisense constructs to restrict expression of seprin in MDA - 436 and utilize sense constructs with MDA - MB - 231 to see if we can stimulate its invasive phenotype.

Conclusions

Research carried out by this laboratory and others on the integral membrane gelatinase we refer to as " seprin " over the past 2 years indicates that the biological function (s) of this protein may be more complex than originally anticipated. Clearly, the expression of this protein is not restricted to metastatic breast cancer and melanoma cells. Our first priority is to determine whether LOX seprin has an identical primary structure to that reported for FAP or that they are closely related proteins but not identical. We should be able to answer this question in the very near future. If there are any significant differences (ie insertions or deletions etc), then it will be important to determine how this difference(s) affects their biological activities. In addition, if a difference in sequence is apparent then we will want to confirm the sequence of seprin in breast carcinoma cell lines like MDA - 436 as well.

Another relevant finding concerning seprin/FAP is that its expression appears to correlate with cell proliferation. For example, FAP is generally only expressed on proliferating but not resting fibroblasts and on activated stromal fibroblasts of various carcinomas in which the stromal compartment can comprise up to 90 % of the tumor mass (Rettig et al., 1993). Thus seprin/FAP may serve as a marker for cell proliferation and/or contribute to the process itself. Therefore transfection experiments involving sense and antisense constructs will be evaluated for the affects of seprin expression not only on invasiveness but also on cell proliferation as well.

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